Standard Procedures for Aquatic Animal Health Inspections

Developed and Edited by:

United States Fish and Wildlife Service
And
American Fisheries Society – Fish Health Section

Chapter 4

Virology

4.1 Introduction

The procedures described in this chapter are specifically for the detection of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Largemouth Bass Virus (LMBV), *Oncorhynchus masou* Virus (OMV), Spring Viremia of Carp Virus (SVCV), Viral Hemorrhagic Septicemia Virus (VHSV) and White Sturgeon Herpesvirus (WSHV).

The initial detection method for all of these viruses is by observing cytopathic effect (CPE) in cell culture using virus isolation procedures. The presence of IHNV and VHSV may be confirmed using either serum neutralization or polymerase chain reaction (PCR) techniques. The presence of IPNV and SVCV is confirmed by serum neutralization. The presence of LMBV and ISAV is confirmed using PCR. OMV and WSHV suspect cultures will be sent to an appropriate laboratory for confirmation.

These procedures may also detect other replicating agents not listed here. When this occurs, every attempt will be made to complete the identification of the organism. Some of these viruses may occur in combination and the finding of one agent will not preclude following procedures that may identify other agents.

If one of these viruses or an unknown replicating agent is found, the proper parties and authorities will be notified in a timely manner and at least one representative sample of each isolate should be archived at -70°C to be used for future reference.

Blind passage of samples not exhibiting CPE after 14 days of primary incubation is included in these procedures to determine if it provides a significant increase in detection of viral agents. It is requested that laboratories using these procedures summarize their findings of primary and blind passage detections by virus and provide it to the Handbook Revision and Oversight Committee annually. If the data shows that blind passage of these samples is not providing a sufficient increase in viral detection, it will be removed from the procedures.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

4.2 Selection of Appropriate Cell Lines

Selection is based on the ability of the cell lines to detect the viruses of interest and, whenever possible, utilizing cell lines capable of detecting multiple different viral agents to increase the efficiency of the laboratory procedures. At the minimum, one cell line with high sensitivity for the virus of interest will be for all samples and at least two cell lines should be used to maximize the detection of viral agents present in the samples. (General references: Bouchard, 1999; OIE, 2000; Plumb, 1999b; Thoeson, 1994; Wolf, 1988)

A. General Considerations

1. All viral testing will utilize cell lines obtained directly from the American Type Culture Collection (ATCC) when available.

2. At the minimum, all cell lines should be tested annually for viral sensitivity and mycoplasma infection.

3. Work with only one cell line at a time.

4. Aseptic technique is required for cell culture work.

B. Cell Line Sensitivities

1. The EPC cell line provides high sensitivity for IHNV, SVCV and VHSV.

2. The SHK-1 cell line provides high sensitivity for ISAV.

3. The FHM and BF-2 cell lines both provide high sensitivity for LMBV.

4. CHSE-214 cell line provides high sensitivity for IPNV and OMV.

5. The WSS-2 cell line provides high sensitivity for WSHV.

Table 4.1 - Recommended cell lines to detect target viruses

Virus[a]	Cell Line	Common Name	ATCC[b] Designation
IHNV	Epithelioma Papulosum Cyprini	EPC	
IPNV	Chinook Salmon Embryo	CHSE-214	CRL-1681
ISAV	Salmon Head Kidney	SHK-1	
LMBV	Fat Head Minnow	FHM	CCL-42
	or Bluegill Fry	BF-2	CCL-91
OMV	Chinook Salmon Embryo	CHSE-214	CRL-1681
SVCV	Epithelioma Papulosum Cyprini	EPC	
VHSV	Epithelioma Papulosum Cyprini	EPC	
WSHV	White Sturgeon Spleen	WSS-2	

[a] Viruses: IHNV- Infectious Hematopoietic Necrosis Virus; IPNV- Infectious Pancreatic Necrosis Virus and other related birnaviruses; ISAV – Infectious salmon anemia virus; LMBV – Largemouth Bass Virus and other related iridoviruses; OMV - *Oncorhynchus masou* Virus; SVCV – Spring viremia of carp; VHSV - Viral Hemorrhagic Septicemia Virus; WSHV - White Sturgeon Herpesvirus.
[b] American Type Culture Collection, Rockville, MD Certified Cell Lines

4.3 Cell Culture

Standard animal cell culture techniques are used with adaptations for fish cell lines when necessary i.e., incubation temperature. Normal appearing cultures composed of rapidly dividing cells will be used for all assay work. Cells will be routinely subcultured to maintain healthy cells, approximately once every two weeks for most cell lines, or split weekly for seeding plates. Aseptic technique is required when working with any cell line. Only one cell line is worked with at a time. (General references: Freshney, 1983; Jakoby, 1979; Merchant, 1964; Rovozzo, 1973; True, 2000)

A. Subculture Procedures for Flasks

1. Suggested split ratios and seeding rates are given in Table 4.2.

2. Remove tissue culture medium by decanting off fluid.

3. Slowly add Trypsin-Versene (EDTA) (4.9.E) solution and rock the flask gently for 1 minute. In a 75 cm^2 flask a volume of 3-4 mls is sufficient to coat cells.

4. Decant again.

5. Carefully observe the cell layer and repeat steps 4.3.A.3 and 4 as necessary until cells start detaching from flask. Dislodging of the cells may be completed by sharply striking the edge of the flask against the heel of a hand.

6. Add tissue culture medium to neutralize the trypsin. In a 75 cm^2 flask, 10 mls is sufficient.

7. Triturate to break up cell clumps and add an appropriate volume of fresh tissue culture medium for transferring to other flasks. Enumeration of cells in the suspension may be done at this time to determine the necessary volume to transfer. (4.A2)

8. Aspirate and dispense into new flasks. The sub-cultivation ratio is generally 1:4 to 1:6. Following manufacturers recommendations, bring total volume in each new flask up to the acceptable level with the appropriate tissue culture medium. For a 75 cm^2 flask this will usually be about 20 mls. MEM-10/Hepes (4.9.G) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with L-15 (4.9.H).

9. Incubate flasks at room temperature (20-25°C) until they reach confluence and then incubate at the appropriate temperature for that cell line.
    a. SHK-1 and CHSE-214 cells should be held at 15°C;

    b. WSS-2 cells should be held at 20°C;

    c. FHM and BF-2 cells should be held at 25°C;

    d. EPC cells may be held at 15-25°C.

Table 4.2 - Seeding Guidelines for the Subculture of Fish Cell Lines

CELL LINE		Suggested	INCUBATION TEMP (°C)	
Common Name	Nominal split Ratio	Seeding Rate (per cm^2)	Suggested	Range
BF-2	1:2 - 3	100,000	25 – 30	20 - 30
CHSE-214	1:3 - 6	100,000	15 - 20	4 - 27
EPC	1:3 - 6	250,000	15 - 25	15 - 30
FHM	1:4 - 6	250,000	25 - 30	0 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	1:2 - 3	250,000	15 - 20	15 - 20

B. Seeding Procedures for Plates

1. Monolayers on plates are prepared approximately 24 to 48 hours prior to inoculation with the sample.

2. A flask of visually healthy cells approximately 7-10 days old is selected and trypsinized as described previously. After neutralizing the trypsin with tissue culture medium, the total volume in the flask is adjusted to provide a cell concentration appropriate for the seeding rate listed in Table 4.2 and the area of the wells to be seeded. The appropriate volume of the cell suspension is then pipetted into each well of the plate.

   Example: When seeding EPC cells in a 24 well plate, 0.5 mls of a 1 X 10^6 cells per ml suspension is dispensed per well.

3. At a minimum, control wells are included in each plate set and should be included on each plate whenever possible. Control wells are made by dispensing tissue culture medium into plate wells that contain normal looking cell monolayers that have not been inoculated with a sample. These wells are observed during the incubation along with the sample wells for abnormalities that may arise due to media or cell problems. A plate set refers to the group of plates seeded from a single flask at the same time.

4. Incubate plates overnight at room temperature (approximately 20-25°C).

4.4 Sample Processing Procedures

Tissue processing for viral culture is described below. Ingredients and preparation procedures for buffers and other solutions/media are listed in section 4.9. (General references: Amos, 1985; OIE, 2000; Rovozzo, 1973; True, 2000; Wolf, 1988)

A. General Considerations

1. As during sampling and transport, care is taken to protect tissues and fluids from exposure to UV light and temperatures lethal to the viruses of interest.

2. During sample processing, dilution levels are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

3. Following inoculation of test media, remaining tissue/fluid products are kept at 4°C until all assays are completed. Subsequent to the completion of the assays, all material is decontaminated and discarded.

B. Processing of Kidney and Spleen Samples

1. If transport medium (2.3.C.2) is used, it is poured off and disinfected before discarding. Tissue samples are weighed to the nearest 0.1 g and sterile sample dilution medium (4.9.A) is added to make a dilution of 1:10 to 1:100 (w/v). Unless there is a high potential for cell toxicity from the sample, the 1:10 dilution will be used.

2. Tissues are homogenized and a measured amount of homogenate is pipetted into a sterile tube for centrifugation.

3. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

4. An aliquot of supernatant (for virologic evaluation) is transferred to a tube containing an equal amount of antibiotic incubation medium (anti-inc) (4.9.B or 4.9.C). Sample dilution is now 1:20 volume/volume.

Tubes are vortexed and incubated for 2 hours at 15°C or 12-24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures (4.5.A).

C. Processing of Coelomic (Ovarian) Fluid Samples

1. An equal volume from each ovarian fluid sample is pipetted into a sterile tube for centrifugation.

2. Centrifuge the tubes at 2000-3000 X g for 15 minutes at 4°C.

3. Undiluted ovarian fluid may be used to inoculate cell cultures as described in the inoculation procedures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts anti-inc)(4.9.B or 4.9.C) may be made.

4. If a dilution is made, tubes are vortexed and incubated for 2 hours at 15°C or 12-24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures.

4.5 Screening Method for Viral Isolation

The initial detection or screening method used for all listed viral agents is the observation of CPE in cell culture. Standard viral propagation techniques include plate inoculation and incubation for a minimum of 14 days with re-inoculation of samples exhibiting CPE. Blind passage of samples not exhibiting CPE is included to optimize detection of low titer and/or slow growing viruses. To maximize detection of viral agents, samples should be inoculated on at least two different cell lines. (General references: Amos, 1985; OIE, 2000; Bouchard, 1999; Plumb, 1999b; Rovozzo, 1973; True, 2000; Wolf, 1988)

A. Plate Inoculation Procedures for Primary Culture

1. General Considerations
    a. All cell monolayers to be inoculated are to be at least 80% confluent, approximately 24 hours old, and visually healthy.

    b. Tissue culture plates are identified by labeling with the cell line, date of inoculation, and sample information.

    c. Aseptic technique is required.

2. Tissue culture medium is decanted from plates.

3. Inoculate with replication at least 2 cm^2 of cell monolayer with a minimum of 100µl from each sample.

    Example: If using 24 well plates (2 cm^2/well), 100µl of each sample would be inoculated onto each of two wells of the plate.

To allow for viral adsorption, incubate plates for 1 hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.
    a. Incubation temperature for IPNV, IHNV, VHSV, ISAV, and OMV is 15°C.

    b. Incubation temperature for WSHV, SVCV, and LMBV is 20-25°C.

5. Dispense an adequate volume of the appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (4.9.F) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with Leibovitz's L-15 (4.9.H). If using 24 well plates, 0.5 ml of media is adequate.

6. Seal each plate.

7. Incubate plates at the appropriate temperature for a minimum of 14 days.
    a. Incubate for IHNV, IPNV, ISAV, OMV, and VHSV at 15°C.

    b. Incubate for WSHV at 20°C.

c. Incubate for LMBV and SVCV at 20-25°C.

8. Monitor cells at least twice per week for cytopathic effect (CPE). CPE is defined as any morphological change that cells may demonstrate in response to viral or toxic agents. It may range from foaming of the cytoplasm to focal clumping or local destruction of cells. Examples of the appearance of normal cell line monolayers and the CPE typical of these viruses are shown in Figures 4.1-4.12.

9. Re-inoculations are made from representative wells exhibiting CPE on these primary inoculations and from at least one well of all samples not exhibiting CPE (blind passage) according to the procedure in 4.5.B.

B. Re-inoculation Procedure

1. General Considerations:
   a. Re-inoculation of wells showing toxicity and to confirm the presence of virus with typical CPE may be performed on individual wells at any time during the primary incubation. These will be maintained as individual samples and plated with replication during the re-inoculation procedure.

   b. Blind passage from at least one well of all samples not exhibiting CPE will be performed after 10-14 days of incubation of the primary culture. It is suggested that the wells remaining on this plate be left intact and observed for at least another 7-10 days for a total initial incubation period of 21 days. These samples may be combined in up to a 5 pool sample (representing up to 25 fish) and plated with replication during the re-inoculation procedure.

      Example: On a lot inspection using 5 fish pools and 24 well plates, 2 of the 12 samples exhibit CPE at day 5 and re-inoculation is performed as described below. The remaining 10 samples (20 wells representing 50 fish) show no evidence of CPE after 14 days of primary incubation and re-inoculation is performed by combining one well from each of 5 samples and inoculating this pooled sample onto 2 wells of a 24 well plate. This is repeated with the other 5 samples using a total of 4 wells on the re-inoculation plate.

   c. As in the initial tissue processing, sample dilution levels for re-inoculation are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

   d. Aseptic technique is required.

2. Using a pipette, stir and scrape the bottom of the well to be subcultured to dislodge the cell layer.

3. Aspirate the fluid and cell debris from the well and place in a sterile tube for centrifugation.

4. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

5. Remove a measured amount of supernatant and place in a separate sterile tube for dilution.

6. Dilute Samples:
    a. For wells exhibiting CPE, use the lowest dilution possible for re-inoculation not to exceed 1:100 using sample dilution medium (4.9.A).

    b. For blind passage samples, up to a 1:5 dilution may be made.

7. If bacterial or fungal contamination is present, the sample should be filtered through a 0.45µ filter before inoculation onto the plate.

8. Use the appropriate amount of each of these solutions to inoculate a new plate as described in the Plate Inoculation Procedure (4.5.A).

9. Monitor these re-inoculation plates at least twice per week for CPE. Total incubation time for both the primary and re-inoculation or blind pass samples is 28 consecutive days.

> **Example:** If the blind pass is performed on day 14 of the primary incubation, the re-inoculation plate is observed for at least an additional 14 days. If the blind pass is performed on day 10, the re-inoculation plate is observed for at least 18 days.

10. Results:
    a. **If no CPE is noted after the 28 day combined incubation period with no apparent problems in the assay, samples are reported as negative and may be discarded using the proper decontamination procedures.**

    b. If CPE occurs at any time during this assay, it is considered a **PRESUMPTIVE positive** result and the identification of the virus should be confirmed by the appropriate method.

4.6 Identification of Viruses

Methods used for confirmation must have high specificity for the agents they are used to identify. Serum neutralization has long been used as a standard for viral identification and Polymerase Chain Reaction (PCR) procedures have recently been developed for many of the listed viruses. Although other methods exist to identify some of the listed viruses, these two methods have been selected for confirming the presence of all of these agents except OMV and WSHV. The identification of IHNV, VHSV, IPNV and SVCV may be confirmed using serum neutralization. The identification of IHNV, VHSV, LMBV, and ISAV may be confirmed by PCR. OMV and WSHV suspect cultures will be sent to an appropriate reference laboratory for confirmation.

A. Infectious Hematopoietic Necrosis Virus (IHNV) - Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the Novirhabdovirus genus of the Rhabdoviridae family. IHNV has a wide geographic range that includes North America, Europe and the Far East. The virus is primarily found in salmonids with rainbow trout fry being highly susceptible to disease. Older fish are more resistant to infection but may become carriers. Transmission is primarily horizontal but cases of egg associated transmission have been recorded as well as transmission by fomites. The virus may be shed in ovarian fluid and excretory products such as feces and may also be isolated from the kidney, spleen, encephalon, and digestive tract of clinically ill fish. Under natural conditions, most clinical disease from IHNV is seen in fry when water temperature is between 8-15°C with fish exhibiting darkening of the skin, ascites, exophthalmia, and petechial hemorrhages internally and externally. Degeneration and necrosis of the hematopoietic tissue in the kidney is thought to be the actual cause of mortality. (Egusa, 1991; OIE, 2000; Wolf, 1988)

Screening method:

a. Cell culture on EPC cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IHNV.**

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers

are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. Polyethylene glycol (PEG) has been used to aid in the visualization of plaque formation but is not necessary to detect IHNV. (Batts, 1989) See Figures 4.1 and 4.2.

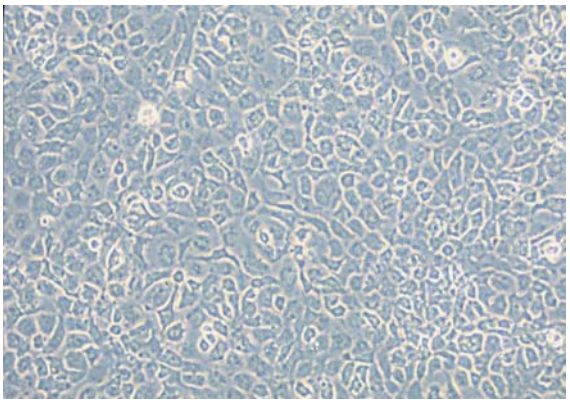


Figure 4.1 Normal EPC monolayer
Photo Courtesy of Jim Winton, USGS

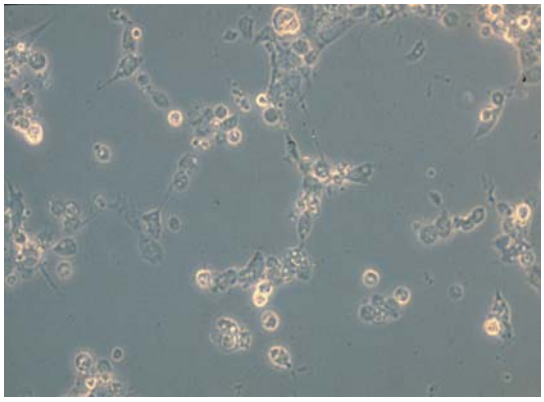


Figure 4.2 CPE typical of a rhabdovirus on EPC monolayer
Photo Courtesy of Jim Winton, USGS

ii. The methods below may be used to confirm that the cause of the CPE is due to the presence of IHNV.

2. Confirmation methods for IHNV:
   a. Serum Neutralization method: See section 4.7 for the general procedure.
      i. Use the cell line on which the initial CPE was produced.

      ii. Incubate plates at 15°C

   b. Polymerase Chain Reaction (PCR) method for confirmation of IHNV (Modified from Arakawa, 1990): The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
      i. Extraction of RNA from cell culture fluid (Heat RNA release method)
         1. Dilute cell culture fluid 1:50 in molecular grade RNase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

         2. Heat tubes to 95°C for 2 min in a water bath, heat block, or thermocycler.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself)

4. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

ii. Production of DNA by Reverse Transcription and Amplification by First Round PCR

1. QA/QC (see Chapter 6 for Specific QA/QC considerations for PCR)

2. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

3. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls).

4. First Round Primers for IHNV:
   a Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'

   b Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'

5. First Round Thermocycler Program for IHNV
   a Pre-dwell at 50°C for 15 minutes

   b Preheat or "Jumpstart" sample to 95°C for two minutes.

   c 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   d Post dwell at 72°C for 7 minutes.

   e Hold samples at 4°C after cycling is complete.

iii. "Nested" Second Round PCR for IHNV

1. QA/QC (see Chapter 6 for Specific QA/QC considerations for PCR)

2. Again use Worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

3. Second Round Primers for IHNV:
   a Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'

   b Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'

4. Second Round Thermocycler Program for IHNV
   a. Preheat or "Jumpstart" sample to 95°C for two minutes.

   b. 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   c. Post dwell at 72°C for 7 minutes.

   d. Hold samples at 4°C after cycling is complete.

   PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

iv. Visualization of PCR Product by Electrophoresis (See 6.3.C)
   1. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
      a. **Bands occurring at the 786 bp location in the First Round Assay and the 323 bp location in the Second Round Assay are confirmatory for IHNV and are reported as POSITIVE.**

      b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IHNV.**

   2. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

B. Infectious Pancreatic Necrosis Virus (IPNV) - Infectious Pancreatic Necrosis Virus (IPNV) is a nonenveloped icosahedral shaped bi-segmented double-stranded RNA virus belonging to the Aquabirnavirus genus of the Birnaviridae family. There are many viruses in the Aquabirnavirus group most of which have not been shown to cause disease in fish so care must be taken to confirm that the virus present is IPNV. IPNV has a wide geographic range that includes North and South America, Asia, and Europe. It is very stable under a wide range of environmental conditions and is capable of surviving for several days in both fresh and saltwater. It is resistant to a wide range of chemical disinfectants including ether, chloroform, and quaternary ammonium compounds but is deactivated by 70% ethanol. Isolates display wide antigenic diversity and virulence. There are two sero-groups that do not cross-react in serum neutralization tests with the majority of strains belonging to sero-group A. IPNV has been isolated from several species of marine and freshwater fish and shellfish. Acute catarrhal enteritis has primarily been seen in salmonid fry and fingerlings with initial mortality occurring in the more robust individuals. Fish that survive the disease may become asymptomatic carriers and shed the virus through the feces and sex products. IPNV may be transmitted vertically as well as horizontally. (Bruno, 1996; Egusa, 1991; OIE, 2000; Roberts, 1982; Wolf, 1988)

1. Screening method:
    a. Cell Culture on CHSE-214 cell line incubated at 15°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IPNV.**

    d. If CPE typical of IPNV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
        i. The appearance of CPE typical of IPNV is described as stellate shaped plaques with spindle-shaped cells. Some of the cells within the plaque will exhibit nuclear pyknosis (nuclei shrink in size and chromatin condenses) with other cells appearing normal. See figures 4.3 and 4.4. Typically, positive cultures result in rapidly lytic CPE but some cells may survive and reform a normal looking monolayer.

        ii. The serum neutralization method is used to confirm the cause of the CPE is due to the presence of IPNV.

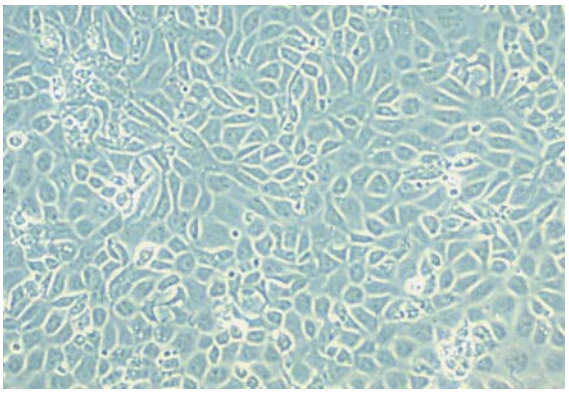


Figure 4.3 Normal CHSE-214 monolayer
Photo Courtesy of Jim Winton, USGS

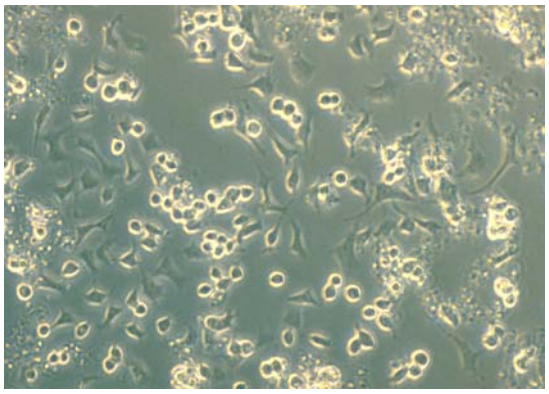


Figure 4.4 CPE typical of IPNV on CHSE-214 cells
Photo Courtesy of Jim Winton, USGS

2. Confirmation method for IPNV - Serum Neutralization method: See section 4.7 for the general procedure.
    a. Use the cell line on which the initial CPE was produced.

    b. Incubate plates at 15°C

C. Infectious Salmon Anemia Virus (ISAV) - Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus most likely belonging to the Orthomyxovirus genus of the Orthomyxoviridae family. Disease is mostly seen in Atlantic salmon in salt water in the spring and fall associated with rapidly changing water temperature. Characteristics of the disease include anemia, ascites, petechial hemorrhages on the peritoneal surface and perivisceral fat, and congestion of the liver, spleen, kidney and upper digestive tract. The virus has been isolated from Atlantic salmon, Rainbow and Brown or sea trout, and Atlantic herring from Europe and the Atlantic coast of North America. (Bruno, 1996; OIE, 2000)

1. Screening method:
    a. Cell Culture on SHK-1 cell line incubated at 15°C. (Bouchard, 1999)

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for ISAV.**

    d. If CPE typical of ISAV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**
        i. The appearance of CPE typical of ISAV is described as plaques of vacuolated cells that round up and loosen from the growth surface. It

may progress to involve the entire cell sheet with only small rounded, refractile and necrotic cells observable. See Figures 4.5 and 4.6.

ii. The Polymerase Chain Reaction (PCR) method is used to confirm the cause of the CPE is due to the presence of ISAV.

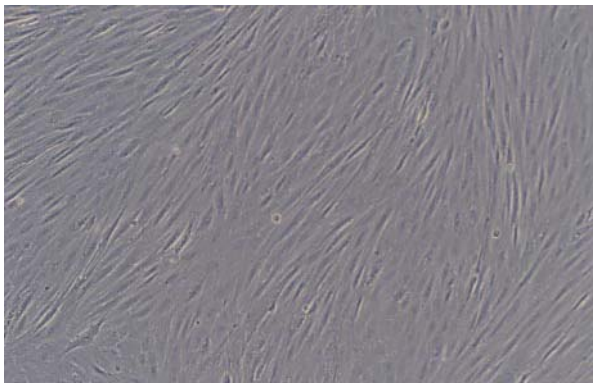


Figure 4.5 Normal SHK-1 monolayer
Photo Courtesy of Jim Winton, USGS

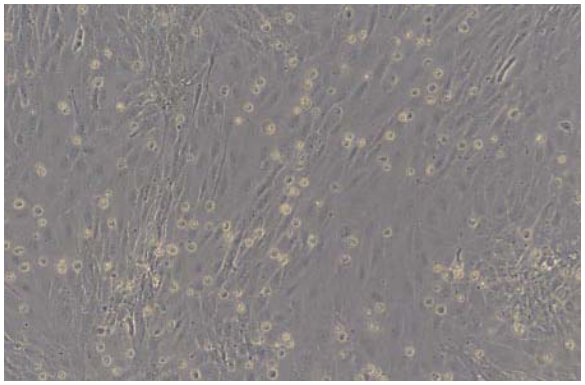


Figure 4.6 CPE typical of ISA on SHK-1 cells
Photo Courtesy of Jim Winton, USGS

2. Confirmation method for ISAV - Polymerase Chain Reaction (PCR) (Modified from Bouchard, 1999) - The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
    a. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method)
        i. Dilute Cell Culture fluid (with some cell scrapings) 1:50 in molecular grade RNAase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

        ii. Heat to 95°C for 2 min in a heat block, water bath, or thermocycler.

        iii. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself)

        iv. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

    b. Formation of DNA by Reverse Transcription and Amplification by PCR
        i. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

ii. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

iii. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls and empty slots in the assay).

iv. Primers for ISAV:

1. Forward: 5'-GGC TAT CTA CCA TGA ACG AAT C-3'

2. Reverse: 5'-TAG GGG CAT ACA TCT GCA TC-3'

v. Thermocycler Program for ISAV

1. Pre-dwell at 42°C for 15 minutes

2. Preheat or "Jumpstart" sample to 94°C for five minutes.

3. 40 cycles as follows:

a. Denaturing at 94°C for 45 seconds.
b. Annealing at 59°C for 45 seconds.
c. Extending at 72°C for 105 seconds.

4. Post dwell at 72°C for 7 minutes.

5. Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

c. Visualization of PCR Product by Electrophoresis (See 6.3.C)

i. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assays.

1. **A band occurring at the 493 bp location is confirmatory for ISAV and the sample is reported as POSITIVE.**

2. **The lack of the appropriate band with no indication of problems with the assay are reported as NEGATIVE for ISAV.**

ii. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

D. Largemouth Bass Virus (LMBV) - Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the Ranavirus genus of the Iridoviridae family. LMBV infection has been found in centrarchid and ecocid populations in the Mid-West and Southeastern United States and has been found experimentally to be associated with mortality in juvenile largemouth bass. (Plumb, 1999a) During an active infection, the virus may be isolated from several tissues including the kidney, spleen, and swim bladder.

1. Screening method:
    a. Cell culture on FHM or BF-2 cell lines incubated at 20-25°C. (Plumb, 1999b)

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial inoculations and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for LMBV.**

    d. If CPE typical of LMBV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**
        i. The appearance of CPE typical of LMBV is described as circular cell free areas with rounded cells at the margins. See Figures 4.7 through 4.10.

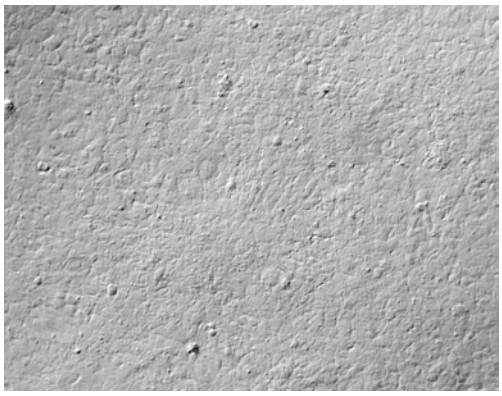


Figure 4.7 Normal FHM monolayer Photo Courtesy of John Grizzle, Auburn University

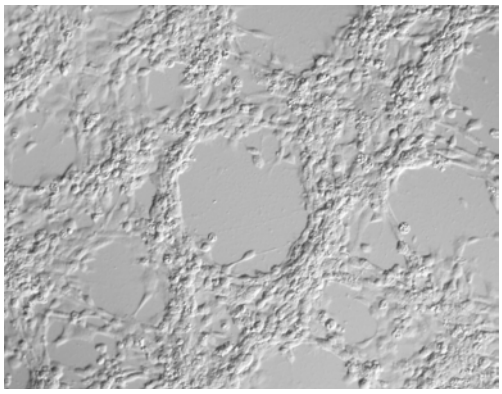


Figure 4.8 CPE typical of LMBV on FHM cells. Photo Courtesy of John Grizzle, Auburn University

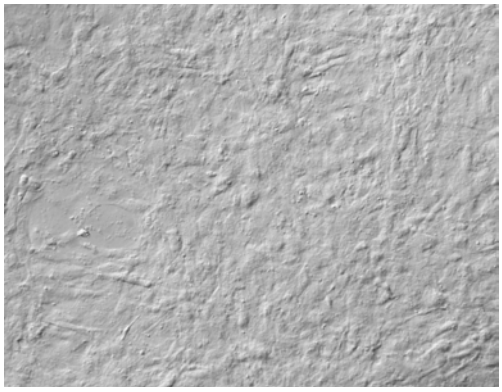


Figure 4.9 Normal BF-2 monolayer Photo Courtesy of John Grizzle, Auburn University

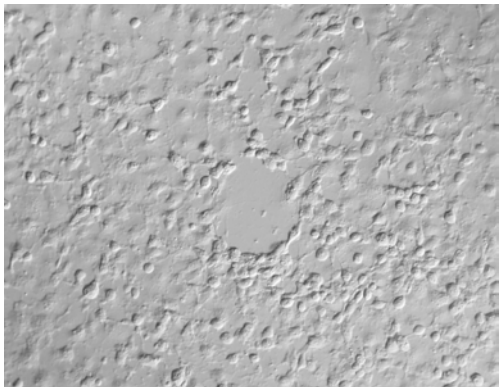


Figure 4.10 CPE typical of LMBV on BF-2 cells. Photo Courtesy of John Grizzle, Auburn University

ii. The Polymerase Chain Reaction (PCR) method is used to confirm that the cause of the CPE is due to the presence of LMBV.

2. Confirmation method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb, 1999b)–This is a DNA containing virus so DNA is extracted from cell culture fluid and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.
    a. Extraction of DNA from cell culture
        i. Supernatant and cells from suspect sample wells are removed and cells lysed in 1ml of 50mM KCL, 10 mM tris-HCL at pH 9.3, and 3 mM Mg Cl_2 containing 0.5% Tween 20.

        ii. Transfer 500µl of lysate to a microcentrifuge tube.

        iii. Add proteinase K solution (5.6.E) to a final concentration of 100µg/ml (example: if stock solution is 20 mg/ml, add 2.5 µl).

        iv. Incubate overnight at 37°C.

        v. Add 500µl of 70% phenol-40% chloroform and vortex.

        vi. Centrifuge at 3-5,000 Xg to separate layers.

        vii. Pipet out the aqueous (top) layer and place in a clean centrifuge tube being careful not to contaminate with any material from the phenol-chloroform layer.

        viii. Repeat steps v.-vii.

        ix. Add to the aqueous layer after the second extraction 50µl of potassium acetate and 1 ml of absolute ethanol. Mix gently.

x. Hold the sample at -20°C for at least 10 minutes to precipitate the DNA.

xi. Centrifuge the sample at 3-5,000 Xg for 30 minutes.

xii. Decant the alcohol solution, add 500µl of 70% ethanol, and mix by inverting the tube.

xiii. Centrifuge at 3-5,000 Xg for 5 minutes and decant off the alcohol.

xiv. Resuspend the DNA pellet in 200µl of TE buffer (10 mM tris-HCL at pH 8.0 and 1mM EDTA).

b. Amplification of LMBV DNA:

i. General QA/QC Considerations (see 6.2 for Specific QA/QC considerations for PCR):

ii. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

iii. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

iv. Primers for LMBV:

1. Forward: 5'-GAC TTG GCC ACT TAT GAC-3'

2. Reverse: 5'-GTC TCT GGA GAA GAA GAA-3'

v. Thermocycler program for LMBV

1. Pre-dwell sample to 94°C for 5 minutes.

2. 30 cycles of the following regime

a. Denaturing at 94°C for 60 seconds.
b. Annealing at 45°C for 60 seconds.
c. Extending at 60°C for 120 seconds.

3 Post dwell at 72°C for 2 minutes.

4 Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

c. Visualization of PCR Product by Electrophoresis (6.3.C) - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.
    i. **A band occurring at the 495 bp location is confirmatory for LMBV and the sample is reported as POSITIVE.**

    ii. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for LMBV.**

d. Photograph the gel (6.3.G) - **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

E. Oncorhynchus Masou Virus (OMV) -

Oncorynchus Masou Virus (OMV) is an enveloped double-stranded DNA virus belonging to the Herpesvirus genus of the Herpesviridae family. Salmonids are the only fish known to be susceptible to infection with OMV with kokanee being the most susceptible. The geographic range has so far been limited to Japan and Eastern Asia. The initial disease is a septicemia that may cause edema and hemorrhage in fry during which time virus will be shed in the feces and urine and isolated from the liver, kidney, and spleen. Several months later, survivors may develop epithelial tumors around the mouth and fins with virus being easily isolated from these lesions. Most disease is seen in water temperatures below 14 °C and although OMV may be isolated from ovarian fluid at spawning, as long as eggs are disinfected after fertilization, transmission is by the horizontal route. (OIE, 2000; Wolf, 1988)

1. Screening method:
    a. Cell culture on CHSE-214 cell line incubated at 15°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for OMV.**

    d. If CPE typical of OMV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
        i. The appearance of CPE typical of OMV is described as the formation of rounded cells which progress to marked syncytia and eventual lysis of the entire cell sheet. See Figures 4.11 and 4.12.

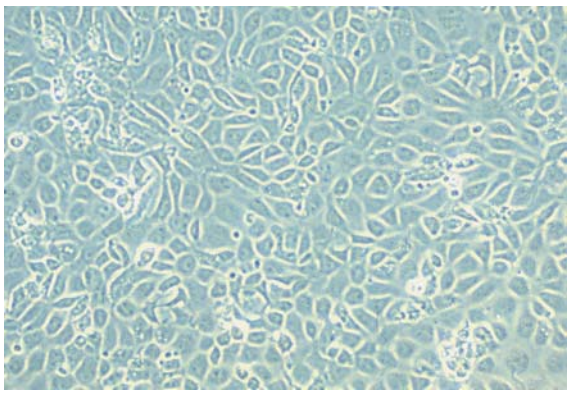


Figure 4.11 Normal CHSE-214 monolayer
Photo Courtesy of Jim Winton, USGS

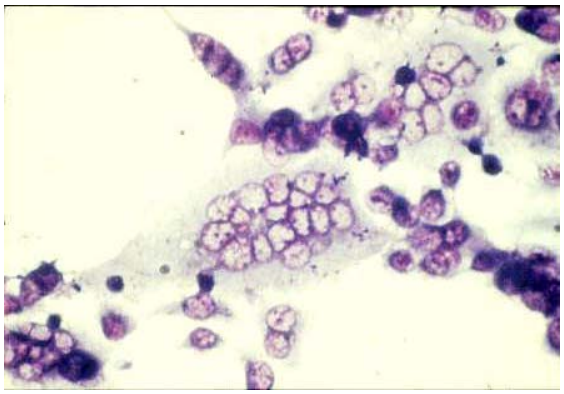


Figure 4.12 CPE typical of OMV on CHSE-214 cells
Photo Courtesy of Mamoru Yoshimizu, Hokkaido University

ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of OMV.

2. Confirmation method:
   a. OMV is considered an exotic pathogen in the United States and many regulations prohibit the maintenance of live virus for positive controls by serological methods. A PCR method will be available soon for identification of OMV but at the present time OMV suspect samples must be sent to a reference laboratory capable of confirming this virus.

   b. A laboratory capable of confirming the identity of OMV is the Laboratory of Microbiology, Hokkaido University, 3-1-1 Minato-cho, Hokodate, Hokkaido 041-0821, Japan, Phone/fax: (81.138) 40.88.10

F. Spring Viremia of Carp Virus (SVCV) - Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the Vesiculovirus genus of the Rhabdoviridae family. The geographic range includes European countries that experience low water temperatures during the winter and has recently been isolated in the USA. The host range includes Pike and Cyprinids with the common carp being the principle host. It causes disease in Cyprinids of all ages characterized by hemorrhages on the skin, gills, and viscera. Mortality is usually seasonal, often most severe in the spring or early summer during rising water temperatures. SVCV may be found in ovarian fluid at spawning but transmission is primarily horizontal and may involve passive transfer by parasites such as the louse and leech. (OIE, 2000; Wolf, 1988)

1. Screening method:
   a. Cell Culture on EPC cell line incubated at 20-25°C.

   b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for SVCV**.

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.

    ii. The Serum Neutralization method may be used to confirm that the cause of the CPE is due to the presence of SVCV.

2. Confirmation method:
    a. Serum Neutralization method: See section 4.7 for the general procedure.
        i. Use the cell line on which the initial CPE was produced.

        ii. Incubate plates at 20-25°C.

G. Viral Hemorrhagic Septicemia Virus (VHSV) - Viral Hemorrhagic Septicemia Virus (VHSV) is an enveloped bullet shaped single stranded RNA virus belonging to the Novirhabdovirus genus of the Rhabdoviridae family. Disease in Continental Europe is mostly seen in trout, grayling, white fish, pike and turbot and is characterized by edema and hemorrhage due to impairment of osmotic balance. Disease in North America is primarily seen in Pacific herring and pilchard but the virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning adult Coho and Chinook salmon. Fry are most susceptible to disease, which usually occurs at water temperatures between 4-14°C. A carrier state may develop with fish shedding virus in the feces, urine, and sexual fluids as well as being present in the internal organs. Although present in ovarian fluid, vertical transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods. (OIE, 2000; Wolf, 1988)

1. Screening method:
    a. Cell culture on EPC cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for VHSV**.

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.

    ii. The methods below may be used to confirm that the cause of the CPE is due to the presence of VHSV.

2. Confirmation methods for VHSV:
    a. Serum Neutralization method: See section 4.7 for the general procedure.
        i. Use the cell line on which the initial CPE was produced.

        ii. Incubate plates at 15°C

    b. Polymerase Chain Reaction (PCR) method for confirmation of VHSV (Modified from Einer-Jensen K, 1995): The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
        i. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method)
            1. Dilute Cell Culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

            2. Place tubes in heat block at 95°C for 2 min.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself)

4. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

ii. Formation of DNA by Reverse Transcription and Amplification by First Round PCR

1. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

2. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

3. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

4. First Round Primers for VHSV:
   a Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'

   b Reverse: 5'-TTC CGG TGG AGC TCC TGA AG-3'

5. Thermocycler Program for First Round VHSV
   a. Pre-dwell at 50°C for 15 minutes

   b. Preheat or "Jumpstart" sample to 95°C for two minutes.

   c. 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   d. Post dwell at 72°C for 7 minutes.

   e. Hold samples at 4°C after cycling is complete.

iii. "Nested" Second Round PCR for VHSV:

1. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

2. Again use Worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

3. Second Round Primers for VHSV:
    a. Forward: 5'-ATG GGC TTC AAG GTG ACA C-3'

    b. Reverse: 5'-GTA TCG CTC TTG GAT GGA C-3'

4. Thermocycler Program for Second Round VHSV
    a. Preheat or "Jumpstart" sample to 95°C for two minutes.

    b. 25 cycles as follows:
        i. Denaturing at 95°C for 30 seconds.
        ii. Annealing at 50°C for 30 seconds.
        iii. Extending at 72°C for 60 seconds.

    c. Post dwell at 72°C for 7 minutes.

    d. Hold samples at 4°C after cycling is complete.

    PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

iv. Visualization of PCR Product by Electrophoresis (6.3.C)

1. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
    a. **Bands occurring at the 950 bp location in the First Round Assay and the 558 bp location in the Second Round Assay are confirmatory for VHSV and are reported as POSITIVE.**

    b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for VHSV.**

2. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

H. White Sturgeon Herpesvirus (WSHV) - White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the Herpesvirus genus of the Herpesviridae family. WSHV has been found in both feral and captive populations of sturgeon in California and Oregon. WSHV-1 has been found in juvenile cultured white sturgeon less than 10 cm. The susceptibility of other sturgeon species to WSHV-1 is not known at this time. WSHV-2 has been isolated from wild and cultured subadult and adult white sturgeon. A herpesvirus has also been isolated from shortnose sturgeon, although the relationship of this isolate to WSHV-1 or WSHV-2 has not been determined. Infected fish may present with lethargy, emaciation, excessive mucus production, fluid in the gastrointestinal tract and focal skin lesions. Horizontal transmission has been demonstrated with both WSHV-1 and WSHV-2. WSHV-2 has been isolated from ovarian fluid but vertical transmission has not been demonstrated. (LaPatra, personal communication, 2002; Plumb, 1999a)

1. Screening method:
    a. Cell culture on WSS-2 cell line incubated at 20°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for WSHV**.

    d. If CPE typical of a herpesvirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for WSHV.
        i. The appearance of CPE typical of WSHV and other herpesviruses includes the formation of syncytia. See Figures 4.13 and 4.14.

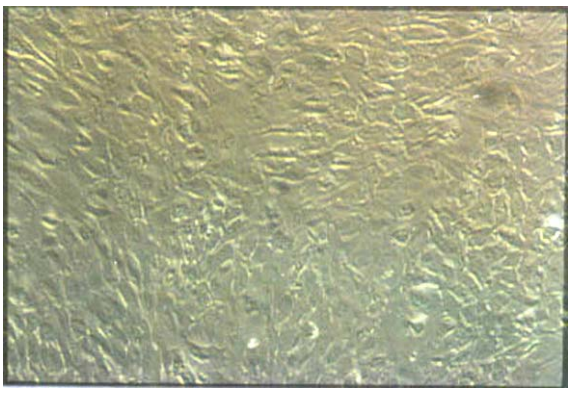


Figure 4.13 Normal WSS-2 monolayer
Photo Courtesy of Scott LaPatra, Clear Springs Foods, Inc

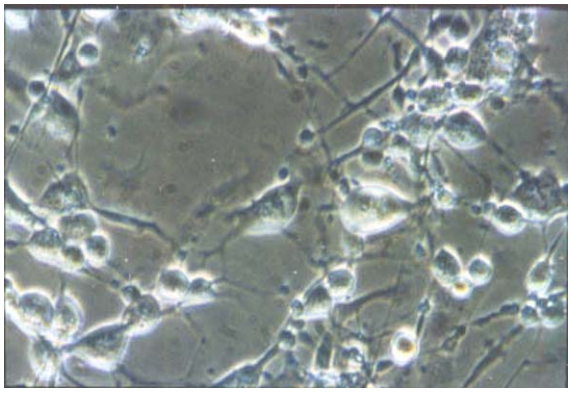


Figure 4.14 CPE typical of WSHV on WSS-2 monolayer
Photo Courtesy of Scott LaPatra, Clear Springs Foods, Inc

ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of WSHV.

2. Confirmation method:
    a. A PCR method has been developed for this virus however the necessary primers are not commercially available at this time. Therefore, suspect samples must be sent to a reference laboratory for confirmation.

    b. A laboratory capable of confirming the identity of WSHV is the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, Phone: 530-752-3411.

4.7 Serum Neutralization

Serum neutralization or plaque reduction assays are serological methods used to confirm the identity of an unknown viral isolate. The procedures involve the use of a known dilution of specific neutralizing antiserum mixed with multiple dilutions of the homologous and suspect virus and subsequent observation of the ability of those viruses to produce CPE when inoculated onto a sensitive cell line. Normal serum from the species of animal used to produce the antiserum (usually rabbit or goat) is used as the negative control to account for nonspecific inhibitors of the virus.

A. Plate Preparation

1. Seed plates (4.3.B) with the appropriate cell line (4.2) 24-48 hours before inoculation with virus.

2. Monolayers should be visually healthy and at least 80% confluent at the time of inoculation.

B. Virus Sample Preparation

1. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^3 to 10^6 plaque-forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per ml of the homologous virus.

2. Dilute the suspect sample and positive control virus to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in sterile HBSS (4.9.A).

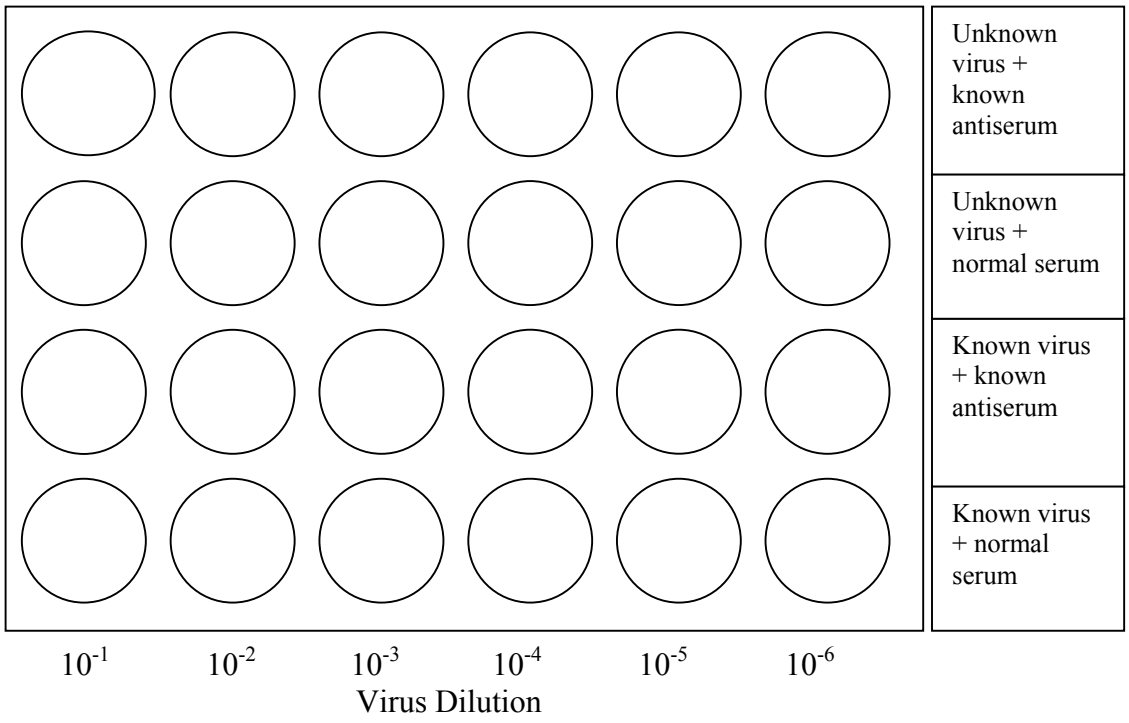
3. Combine equal volumes of each dilution of the suspect sample with the diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour with agitation at the appropriate temperature.
    a. For IHNV, VHSV, and IPNV, incubate at 15°C.

    b. For SVCV incubate at 20-25°C.

4. Inoculate each of these mixtures onto the cell line in which the suspect virus was isolated as indicated in Figure 4.13 below. Incubate at the above temperature for 14 days and observe plates for cytopathic effect (CPE) (See figures 4.1 – 4.12).
    a. **Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification of the suspect virus.**

    b. Alternatively, in a sample mixed with antibody, a titer decrease of 2 $\log_{10}$ indicates neutralization and confirms the identity of the virus.

Figure 1- Diagram for Serum Neutralization Assay using a 24 well plate



C. Preparation of Reference Viruses - Where appropriate, positive controls are produced and frozen for use as needed in the serum neutralization assay.

1. Thawing of Frozen Viral Isolates - Thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.

2. Procedure for Producing Reference Viruses:
    a. Inoculate viral suspensions onto cell culture flasks containing visibly healthy monolayers of the appropriate cell line. This is done in a manner similar to the tissue inoculation described above (4.5.A) using an appropriate volume of inoculum for the flask size. If using a 25cm^2 flask, 0.1 ml of viral inoculum is usually sufficient.
        i. Use EPC cell line for IHNV, SVCV, and VHSV isolates

        ii. Use CHSE-214 cell line for IPNV isolates

    b. To allow for viral adsorption, incubate flasks at the appropriate temperature for 1 hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.
        i. For IHNV, IPNV and VHSV incubate at 15°C.

        ii. For SVCV incubate at 20-25°C.

c. Aseptically dispense an appropriate amount of tissue culture media into the flask. For a 25 cm^2 flask this will be approximately 5 ml.

d. Incubate control sample flasks to allow replication of the viruses
    i. For IHNV, IPNV, and VHSV incubate at 15°C until CPE occurs or for 14 days.

    ii. For SVCV incubate at 20-25°C until CPE occurs or for 14 days.

Procedure for Harvesting the Virus

a. Using aseptic technique, scrape the cell layer from the flask and triturate to break up.

b. Pour fluid and suspended cells into sterile tubes for centrifugation.

c. Centrifuge tubes at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

d. Use supernatant as positive control virus. Any fluid not needed for the assay may be aliquoted into vials and frozen at -70 C.

e. Any supernatant that is not frozen or used for the assay must be decontaminated before it is discarded.

4.8 Glossary

Blind passage - transfer of supernatant and inoculated tissue culture cells which are not demonstrating CPE to another plate containing fresh cells in order to dilute out possible inhibitors of viral expression and/or allow possible early viral replication due to low concentrations of virus particles to progress to detectable CPE.

Closed System - a system of incubating cells that is sealed against the transfer of air, i.e., a sealed flask.

Confluent Monolayer (100%) - a single layer of tissue culture cells in which the cells have filled in all the spaces between them.

Controls

A. **Monolayer control:** tissue culture cells are grown in presence of tissue culture medium. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

B. **Sham control:** diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, tissue culture medium is added. If CPE appears in sham control wells, test is invalidated and must be repeated.

Cytopathic Effects (CPE) - changes in the morphology and metabolism of tissue culture cells. It may be due to viral or toxic agents and the appearance may range from simple foaming of the cytoplasm or focal clumping of cells to complete destruction of the cell monolayer.

FBS - fetal bovine serum taken from unborn calves in utero.

Fomite – an inanimate object such as a net, brush, or clothing, on which a pathogenic microorganism may be transmitted from one animal to another.

Homologous virus – as used in the viral serum neutralization procedure, it is the positive control virus of the same identity used to make the neutralizing antibody.

Monoclonal Antibody (MAb) - antibody produced by tissue culture cell lines derived from the spleen lymphocytes of immunized mice that have been fused (hybridoma) with mouse myeloma tumor cells. Hybridoma cells are cloned to select specific populations of cells, each producing a single antibody against one epitope or antigenic determinant site on one antigen molecule among those used to immunize the mice.

Normal Serum – as used in the viral serum neutralization procedure, it is serum from the same species of animal in which the neutralizing antibody is produced. It is used as a control for any nonspecific viral inhibition that may occur even with a non-homologous virus.

Open System - a system of incubating tissue culture cells that is open to the transfer of air, i.e., a plate. Requires a medium that is buffered against rising pH due to CO_2 loss. Common buffering systems are TRIS and HEPES.

Plaque - a hole or focus of degenerate or dead tissue culture cells in the cell monolayer caused by viral replication. One discrete plaque is assumed to be caused by infection with one infectious particle or aggregate (called one plaque-forming unit = pfu).

Plate set - a group of plates seeded from a single flask at the same time.

Polyclonal Antibody - the entire population of antibodies produced in the sera of immunized animals that are directed against many epitopes on many of the antigenic molecules used for immunization. Most immunogens injected are whole cells or viruses that are composed of many different antigen molecules. Each antigen molecule may have more than one epitope. See "Monoclonal Antibody.

Re-inoculation - transfer of inoculated tissue culture cells and supernatant from one plate to another that contains fresh cells. Used for suspected positive cultures to confirm presence of viral CPE as opposed to toxicity or contamination. Also used to replicate more viruses for storage, etc.

Serum neutralization - antibody molecules in the antiserum neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the corresponding virus (antigen). This prevents virus attachment to and subsequent penetration of host tissue culture cells or virus replication once inside the cell. Neutralization of viruses by antibodies is specific and used to confirm viral identity. Neutralization may be reversible.

Subculture – transfer of tissue culture cells from one container to another for the purpose of forming a new monolayer.

$TCID_{50}$ - denotes fifty percent tissue culture infective dose. This is the reciprocal of the highest dilution of virus that causes CPE in 50% of the wells inoculated with that dilution of infectious materials. This is determined by the Reed and Muench (1938) method.

Tissue Culture-Grade Water - High quality water (low in ions, minerals and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells. De-ionization at greater than 17 Ohms is sufficient to achieve this quality.

Titer - the number of infectious units or plaque-forming units per unit of sample, i.e., per gram or ml.

Toxicity - changes in cell morphology or metabolism caused by toxic substances in the medium or inoculum. This can either cause cell death or interfere with cell metabolism, thereby reducing or preventing replication of the virus. These effects may have arisen through sample toxicity, bacterial or fungal contamination, improper glassware cleaning or improper

media preparation. Usually toxicity can be distinguished from viral CPE by how rapidly it occurs (1 day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus, and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

> **NOTE:** Inoculation of very high-titer suspensions of certain viruses can cause an apparent toxic effect within 24 hours. If there is any doubt to whether disruption of the cell layer was caused by toxicity or CPE, a subculture should be made. This is especially true for some inocula that can produce toxic effects that may take 5-7 days for development.

Triturating - The act of dispersing tissue culture cells for transfer by repeatedly drawing the cell suspension into a pipet and expelling it back into the flask. This should be done until the cells are in clumps of no more than three when examined with an inverted light microscope.

Trypsin - a proteolytic enzyme used to disperse cells and causes their release from the culture surface. Serum proteins neutralize it and its action is slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - ethylene di-amine tetra-acetic acid is a chelating agent involved in causing cells to release from the culture surface.

4.9 Reagents and Media

A. Sample dilution medium - Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 ml
Tissue Culture Grade Water	895.3 ml
$NaHCO_3$ (7.5%)	4.7 ml

Mix. Filter with 0.2 um filter.

B. Antibiotic incubation medium (anti-inc) made with HBSS for sample disinfection

10X HBSS	100.0 ml
Tissue Culture Grade Water	575.0 ml
$NaHCO_3$ (7.5%)	5.0 ml
Penicillin/Streptomycin	160.0 ml
Penicillin G (10,000 units/ml)	
Streptomycin sulfate (10,000 ug/ml)	
Fungizone	160.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix. Filter with 0.2 um filter. Store at 4° C.

C. Antibiotic incubation medium (anti-inc) made with Minimum Essential Medium (MEM-0) for sample disinfection

10X MEM (Eagles Modified Medium)	100.0 ml
Tissue Culture Grade Water	540.0 ml
L-Glutamine (200 mM)	10.0 ml
$NaHCO_3$ (7.5%)	30.0 ml
Tryptose Phosphate Broth	100.0 ml
Penicillin/Streptomycin	160.0 ml
Penicillin G (10,000 units/ml)	
Streptomycin sulfate (10,000 ug/ml)	
Fungizone	160.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix aseptically. Filter with 0.2 um filter. Can store frozen for approximately 3 months. Avoid freeze-thaw cycles, thaw tubes immediately prior to use.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
$KHPO_4$	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Disodium Versenate (EDTA)	0.2 g
Phenol Red (0.5% solution)	2.0 ml
Tissue Culture Grade Water	to 1000 ml

Autoclave and store at room temp.

E. Trypsin-Versene (EDTA)

Trypsin (2.5% solution)	20 ml
Versene (EDTA) (1:5000)	480 ml

Store at -20° C.

F. MEM-5/Hepes (tissue culture medium for all cell lines except SHK-1)

10X MEM	100.0 ml
Tissue Culture Grade Water	810.0 ml
Fetal Bovine Serum	50.0 ml
Sodium Bicarbonate (7.5% solution)	10.0 ml
L-Glutamine (200 mM)	10.0 ml
Hepes Buffer (1M)	15.0 ml
NaOH (1M)	5.0 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

If antimicrobials are included, use 796.0 ml of water above instead of 810.0 and add

Gentamicin (50 mg/ml)	4.0 ml
Fungizone	10.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix aseptically, filter with 0.2 um filter and store at 4° C.

G. MEM-10/Hepes (tissue culture medium for all cell lines except SHK-1)

10X MEM	100.0 ml
Tissue Culture Grade Water	760.0 ml
Fetal Bovine Serum	100.0 ml
Sodium Bicarbonate (7.5% solution)	10.0 ml
L-Glutamine (200 mM)	10.0 ml
Hepes Buffer (1M)	15.0 ml
NaOH (1M)	5.0 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix aseptically, filter with 0.2 um filter and store at 4° C.

H. Leibovitz's L-15 (tissue culture medium for SHK-1 cell line)

1X L-15 with 0.3g/L L-glutamine	1000.0 ml
Fetal bovine serum (5%)	50.0 ml
Gentamicin (50 mg/ml)	1.0 ml
2-mercaptoethanol (0.055 M)	0.7 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix aseptically, filter with 0.2 um filter and store at 4° C.

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4.A1 PCR Worksheets

A. Worksheet A - PCR Sample Data/Log Sheet

PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
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16.			
17.			
18.			
19.			
20.			

B. Worksheet B - Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens

1. Infectious Hematopoietic Necrosis Virus (IHNV)

2. Infectious Salmon Anemia Virus (ISAV)

3. Largemouth Bass Virus (LMBV)

4. Viral Hemorrhagic Septicemia Virus (VHSV)

Worksheet 4.A1.B.1: Infectious Hematopoietic Necrosis Virus (IHNV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	23.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 U/Rx	9 U/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	27.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
Round 1 Product		-	-	2 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for IHNV

	Forward	**Reverse**
1st round	5'-TCA AGG GGG GAG TCC TCG A-3'	5'-CAC CGT ACT TTG CTG CTA C-3'
2nd round	5'-TTC GCA GAT CCC AAC AAC AA-3'	5'-GCG CAC AGT GCC TTG GCT-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	**Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)**	**Weight of agarose (grams)**	**Volume of Buffer (ml)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.2: Infectious Salmon Anemia Virus (ISAV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	26.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	50 pmoles/µl	1 µl	
(-)Primer		50 pmoles/Rx	50 pmoles/µl	1 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for ISAV

	Forward	**Reverse**
1st round	5'-GGC TAT CTA CCA TGA ACG AAT C-3'	5'-TAG GGG CAT ACA TCT GCA TC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.3: Largemouth Bass Virus (LMBV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µl) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	15.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		1.5 mM	25 mM	3 µl	
dNTP's		0.8 mM	10 mM	4 µl	
TMAC		40 µM	100 µM	20 µl	
(+)Primer		50 pmoles/µl	100 pmole/µl	0.5 µl	
(-)Primer		50 pmoles/µl	100 pmole/µl	0.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
DNA Template		-	-	1 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for LMBV

	Forward	**Reverse**
1st round	5'-GAC TTG GCC ACT TAT GAC-3'	5'-GTC TCT GGA GAA GAA GAA-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.4: Viral Hemorrhagic Septicemia Virus (VHSV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	23.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	27.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
Round 1 Product		-	-	2 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for VHSV

	Forward	Reverse
1st round	5'-TCT CTC CTA TGT ACT CCA AG-3'	5'-TTC CGG TGG AGC TCC TGA AG-3'
2nd round	5'-ATG GGC TTC AAG GTG ACA C-3'	5'-GTA TCG CTC TTG GAT GGA C-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

C. Worksheet 4.A1.C - Photodocumentation of the PCR Product Gel

Case Number _____ Date:

Samples

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

4.A2 Cell Enumeration (True, 2000)

Rarely are cells counted during routine propagation of cell lines, however the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 ml, the cell concentration/ml is the average count per square x 10^4. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 min at 500 rpm) and re-suspended in protein-free medium or Hanks salt solution prior to counting.

A. MATERIALS

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipet
Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
Trypsin - EDTA
Pipets 1-ml, sterile, cotton plugged
22 x 22 mm cover-slips

B. PROCEDURE

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface as described in 4.3A.

2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20-50 cells/mm^2 (1 x 10^5 to 2 x 10^5 cells/ml). Dilutions vary depending on age of the cells, cell density and cell aggregation.
3. Aseptically transfer 0.5 ml of the cell suspension into a dilution tube.

Add 0.5 ml Trypan Blue stain (0.1%).

> **Note:** If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

4. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipet to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipet tip and allow each chamber to fill by capillary action. Don't overfill or underfill the chambers.

5. Using a microscope with a 10x ocular and a 10x objective count 10 squares (5 from each chamber) as outlined above.

6. Calculate the # of cells/ml and the total # of cells as follows:

   Cells/ml = x (mean) count per square x 10^4 x Trypan Blue dilution factor

   Total cells in flask = cells/ml x total volume of cell suspension

   e.g., total # cells counted in 10 squares = 300 cells

   x count/square = 300 cells/10 squares = 30 cells

   cells/ml = 30 x 10^4 x 2 (dilution factor)
   cells/ml = 60 x 10^4 cells/ml
   cells/ml = 6.0 x 10^5 cells/ml

   Total cells = 6.0 10^5 cells/ml x 8 ml (original volume cell suspension)
   Total cells = 48.0 x 10^5 cells
   Total cells = 4.80 x 10^6 cells

If the cells/ml calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

ml medium needed = (actual cells/ml) (vol. of cell suspension)/ desired cells/ml

e.g., actual count = 6 x 10^6 cells/ml
desired count = 1 x 10^6 cells/ml
volume of cell suspension = 8 ml
ml medium needed = x

x = ml medium needed = 6 x 10^6 cells/ml x 8 ml / 1 x 10^6 cells/ml

$$\text{ml medium needed} = 48 \times 10^6 \text{ ml} / 1 \times 10^6$$

$$= 48 \text{ ml}$$

Since you have 8 ml already in the flask, you would need to add 40 ml of medium to the flask before splitting to get the recommended seeding cell density for each new culture.